

Phase Variation Analysis of *Coxiella burnetii* during Serial Passage in Cell Culture by Use of Monoclonal Antibodies

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Antigenic changes in *Coxiella burnetii* Nine Mile strain phase I during serial passages in cell culture were analyzed with three groups of monoclonal antibodies (MAbs) against lipopolysaccharide. The MAbs of group 1 did not react with organisms that were passaged over five times, and the MAbs of group 2 did not react with organisms that were passaged over eight times. The MAbs of group 3 reacted with organisms passaged up to 15 times but did not react with phase II cells. These results suggest that *C. burnetii* could be differentiated into four phase states during phase variation.

Coxiella burnetii, the etiological agent of Q fever, has been isolated from a wide range of animals (7). This obligate intracellular bacteria undergoes a transition from a virulent phase (phase I) to an avirulent phase (phase II) upon serial passage in embryonated eggs or cell cultures. This phenomenon, termed phase variation, is analogous to the smooth-to-rough lipopolysaccharide (LPS) transition of gram-negative enteric bacteria (3, 6, 18). In sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), phase I LPS appears as ladder-like bands in the region from 14 to 29 kDa whereas phase II LPS appears as a single band at 2.6 kDa. Phase I LPS contains O-polysaccharide and an outer core in addition to the contents of phase II LPS (1, 2, 21, 22). During phase variation, phase I LPS changes to intermediate-phase LPS and then to phase II LPS. Intermediate-phase LPS shows a decreased number of ladder-like polysaccharide components and is thus considered to be intermediate between phase I and II LPSs (6, 13, 16). This structural change of LPS causes antigenic change of *C. burnetii*. In our laboratory, monoclonal antibodies (MAbs) were produced to analyze the antigenicity of the immunogenic components of *C. burnetii*, LPS, and membrane polypeptide (5, 14). The reactions of the MAbs against LPS suggest that *C. burnetii* exhibits four antigenic forms during phase variation. This is the first report to present the results of a study using MAbs to analyze the phenomenon of phase variation.

C. burnetii Nine Mile strain phase I cells were obtained from the American Type Culture Collection. The Nine Mile strain phase II cells were obtained from J. Kazar. Both organisms were passaged three times in chicken embryo yolk sacs and were then used as the phase I and II cells in this experiment. These organisms were passaged in persistently infected Buffalo Green Monkey (BGM) cells. The medium was replaced at 5-day intervals with fresh medium, and passage was performed 15 days postinfection by transferring the supernatant containing *C. burnetii* to a fresh monolayer of BGM cells. Purification

of the organism was performed as described previously (9). Purified LPSs of cloned Nine Mile strain phases I and II and LPS of Crazy variant, one of the intermediate-phase cells of Nine Mile strain (6), were kindly provided by K. Amano. The hybridomas were obtained from the fusion of spleen cells from BALB/c mice, which had been immunized with formalin-killed Nine Mile strain phase I, and myeloma cells (P3-X63-Ag8.653), as described previously (4, 11, 17, 23). The MAb-producing hybridomas were selected by indirect immunofluorescence assay with the antigens containing uninfected and *C. burnetii*-infected BGM cells. SDS-PAGE was performed with a 15% polyacrylamide gel as separating gel (12). The whole-cell lysate, which had been treated with proteinase K, was used as the LPS antigen of the organisms cultivated in our laboratory (19). Precision protein standards (Bio-Rad, Hercules, Calif.) were used as molecular weight markers. The gel was stained with silver for LPS as described by Hitchcock and Brown (8). Western blotting was done on a polyvinylidene difluoride membrane. The reaction with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (A+G+M) (ICN Pharmaceuticals, Inc.-ICN Products) was visualized with diaminobenzidine tetrahydrochloride, CoCl_2 , and H_2O_2 (20). The sensitivity of the epitope to weak and strong periodate oxidation treatments was tested by an enzyme-linked immunosorbent assay as described previously (15).

Twenty hybridoma cell lines secreting MAbs were obtained from several fusion experiments. Nineteen MAbs that reacted with both whole-cell lysate and the proteinase K-digested antigen of *C. burnetii* were selected for further experiments. These MAbs were divided into three groups, based on their reaction patterns. The MAbs of group 1 (H5A, H45, and H83) mainly reacted with ladder-like bands in the region from 20 to 27 kDa of phase I LPS, and the MAb of group 2 (H21) reacted with ladder-like bands in the region from 15 to 27 kDa of phase I LPS. The MAbs of group 3 (H64, H70, H72, H73, H76, H78, H80, H86, H91, H99, H100, K1, K36, K90, and K98) reacted with the 14-kDa bands of both phase I and Crazy variant LPSs. None of the MAbs reacted with phase II LPS at all (Fig. 1). These results indicate that the MAbs recognize the components contained in Nine Mile strain phase I LPS, O-polysac-

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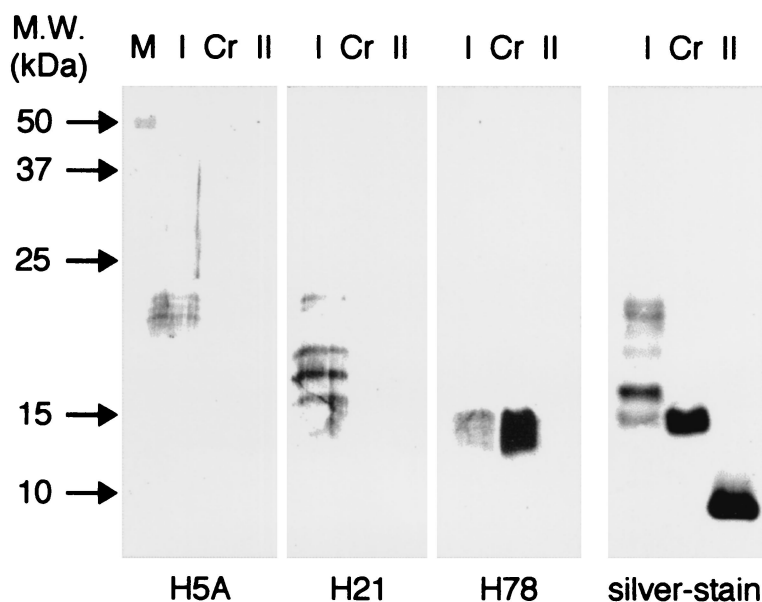


FIG. 1. Profile of purified LPS in Western blotting and silver staining following SDS-PAGE in a 15% acrylamide gel concentration. Purified LPSs of Nine Mile strain phase I (I), Crazy variant (Cr), and phase II (II) are shown in parallel from left to right in each strip. Reactions of the MAbs of groups 1, 2, and 3 (H5A, H21, and H78, respectively) are shown. Molecular mass markers appear on the left (M).

charide chains, and outer-core oligosaccharides (1, 2, 21). The reactivity of the MAbs against weak and strong oxidized antigens showed that the antigens recognized by the MAbs of group 3 are more resistant to periodate oxidation than those recognized by the MAbs of groups 1 and 2 (data not shown). Combined with the chemical analysis of LPS reported previously (2), our findings suggest that the MAbs of groups 1 and 2 recognize O-polysaccharide chains, which mainly contain virenose, and the MAbs of group 3 recognize outer-core

oligosaccharides, which contain galactosamine uronyl- α (1-6)-glucosamine and dihydrohydroxystreptose (2). Further immunochemical analysis would help to determine the sugar constitution of their epitopes and may help to estimate the structure of phase I LPS.

Antigenic changes in Nine Mile strain phase I cells during serial passages in BGM cell culture were partially analyzed by Western blotting. The MAbs of group 1 reacted with organisms that were passaged less than five times, and the MAb of

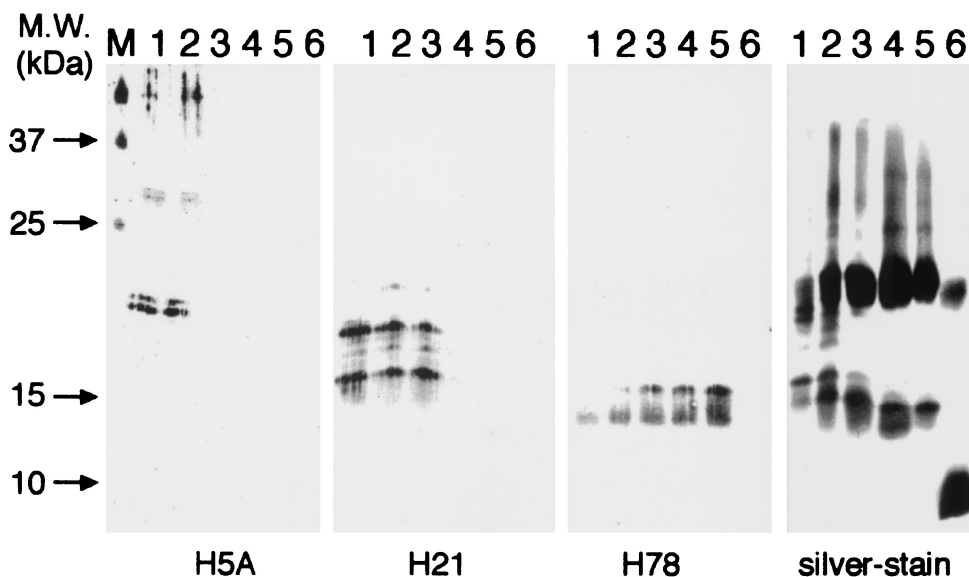


FIG. 2. Change of MAb reactivity with number of passages. LPS banding patterns of each organism were observed using MAb H5A (group 1), MAb H21 (group 2), MAb H78 (group 3), and silver staining. The antigens applied were Nine Mile strain phase I cells passaged three times in egg yolk sac and then passaged in BGM cell culture 0 (lane 1), 3 (lane 2), 6 (lane 3), 9 (lane 4), and 15 times (lane 5) and phase II cells (lane 6). Molecular mass markers appear on the left (M).

group 2 reacted with organisms that were passaged less than eight times. On the other hand, the MAbs of group 3 reacted with organisms passaged up to 15 times. The reaction patterns of representative MAbs of groups 1 (MAb H5A), 2 (MAb H21), and 3 (MAb H78) with phase I cells (passaged in BGM cell culture 0, 3, 6, 9, and 15 times) and phase II cells are shown in Fig. 2. These results suggest that the MAbs of group 1 recognize phase I LPS specifically and that the MAbs of groups 2 and 3 react with intermediate-phase LPS as well. Although it is unclear whether an intermediate-phase cell changes to a phase II cell with subsequent passages in BGM cell culture, our finding suggests that *C. burnetii* phase I decreases its reactivity with the MAbs of group 1 first and then with the MAbs of groups 2 and 3, in that order, during phase variation. With this antigenic difference, *C. burnetii* could be differentiated into four phase states consisting of phases I and II and two intermediate phases, and Crazy variant can be categorized as being in the same phase state as the organisms passaged over nine times. The MAbs obtained in this study will be useful tools for monitoring the progression of phase variation, which are needed for routine laboratory experiments and the diagnosis of Q fever.

The present results indicate that *C. burnetii* LPS has at least four antigenic forms during phase variation. Phase variation is a phenomenon pertaining not only to structural and antigenic change of LPS but also to changes in the virulence and in the serological, biological, and physicochemical properties of *C. burnetii* (10). Further characterization of the variation of these properties will help to define the relation between LPS structure and the phenomena of phase variation, which may help to clarify the role of each LPS component.

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